IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Ian Garner, Michael A. Dalrymple, Donna E. Prunkard, Donald C. Foster
Serial No. : 08/206,176
Filed : March 3, 1994
For : PRODUCTION OF FIBRINOGEN IN TRANSGENIC ANIMALS
Examiner : Stanton, B.
Art Unit : 1804
Docket No.: 93-15
Date : May 8, 1995

Assistant Commissioner of Patents and Trademarks
Washington, D.C. 20231

Declaration of Donna E. Prunkard Under 37 C.F.R. § 1.132

Sir:

I, Donna E. Prunkard, hereby declare as follows:

1. I am an Associate Scientist at ZymoGenetics, Inc., an assignee of the above-identified application.

2. I have read and understand the specification and claims of the above-identified application.

3. The Experiments described in this Declaration were performed by me or by those under my direct supervision.

4. I have read the Office Action dated February 6, 1995 in the subject application, including the rejections under 35 U.S.C. § 103.

5. Experiments were carried out to characterize fibrinogen produced in the milk of transgenic mice. The mice had been generated by microinjection of fibrinogen subunit DNA constructs as disclosed in the subject patent application. Milk collected from seven transgenic female mice was examined for the presence of fibrinogen protein by gel electrophoresis and western blot analyis. To estimate the concentration of fibrinogen subunits in the milk, known amounts of a human plasma fibrinogen standard and the transgenic milk samples were electrophoresed under reducing conditions. A western blot was prepared from the gel and probed with a commercially available rabbit polyclonal anti-fibrinogen antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. Immunoreactive protein was visualized by chemiluminescence and exposure to X-ray film. Band intensities were compared by desitometry. Fibrinogen protein concentration was estimated by comparison of band intensities in transgenic milk samples with the intensities of known plasma fibrinogen standards. Estimates of total fibrinogen production ranged from 40 μg/ml to 1000 μg/ml.

6. Assembly of the fibrinogen subunits into the native hexamer was investigated using a monoclonal antibody that recognized only fully assembled fibrinogen. The antibody did not recognize individual subunits or $\alpha\beta\gamma$ trimers, even when they were electrophoresed under non-reducing conditions. Goat anti-mouse IgG polyclonal antisera conjugated to horseradish peroxidase was used as the second antibody to visualize the protein in a western blot format. Fully assembled fibrinogen hexamer was found to be present only in milk from animals that had been shown (by reducing electrophoresis) to be producing all three fibrinogen subunits. The percentage of total fibrinogen protein present as fully assembled hexamer was estimated by comparing the total fibrinogen protein seen on reducing gels with the amount of assembled hexamer seen on non-reducing

gels. These estimates ranged from 10% to 100%, with the higher levels of assembled fibrinogen seen in animals producing higher amounts of total fibrinogen protein. There was no evidence of fibrinogen degradation in the milk samples.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity of any patent issuing from this patent application.

Donna E. Prunkard

Donna E. Prunkard

Date: 8 May 1995

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EXPRESSION OF FUNCTIONAL HUMAN FIBRINOGEN FROM cDNA CLONES IN BABY HAMSTER KIDNEY CELLS. David H. Farrell*, Eileen R. Mulvihill*, Dominic W. Chung*, and Earl W. Davie. Department of Biochemistry, University of Washington, Seattle, WA and Zymogenetics, Seattle, WA.

Fibrinogen is a plasma protein composed of two α, two β, and two γ chains linked by disulfide bonds. Full-length cDNAs coding for the α, β, and γ chains have been cloned into two mammalian expression vectors along with dihydrofolate reductase as a selectable marker. These expression vectors were co-transfected into a thymidine kinase$^-$ derivative of a baby hamster kidney cell line (BHK-21) and selected with methotrexate. Most methotrexate-resistant colonies secreted immunoreactive fibrinogen into the medium as assessed by immunofilter assays. Clones secreting fibrinogen at levels up to 1-2 mg/liter/24 hours were obtained by stepwise increases in methotrexate concentration. Metabolically-labeled recombinant fibrinogen co-migrated on unreduced SDS-PAGE with authentic human fibrinogen secreted by the human HepG2 cell line. Upon reduction, the α, β, and γ chains also co-migrated with HepG2 fibrinogen. The recombinant fibrinogen was readily incorporated into a fibrin clot in the presence of recalcified human plasma. In addition, reduced SDS-PAGE of the clotted material showed that the α band disappeared and the γ chain shifted mobility to the position of a γ-γ dimer, indicating that the recombinant fibrinogen had been crosslinked by factor XIIIa. These data demonstrate that the transfected BHK cells are capable of producing a properly assembled fibrinogen which is active both in clotting and as a substrate for factor XIIIa. The development of this system has made it possible to examine the complex assembly mechanism of fibrinogen and to express variant forms of fibrinogen in order to study structure/function relationships of the protein.

For reference purposes, please provide 5 keywords that are not in the title of your abstract.

blood
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gene expression
tissue culture

BIOSYNTHESIS OF RECOMBINANT HUMAN FIBRINOGEN IN A MODEL MAMMALIAN CELL EXPRESSION SYSTEM IS LIMITED BY A POST-TRANSCRIPTIONAL PROCESS

Donna Prunkard and Don Foster
ZymoGenetics, Inc., Seattle, Washington.

Fibrinogen is a plasma protein which is secreted by the liver as a multimer composed of two each of three different polypeptide chains, α, β and γ. Human liver HepG2 cells are known to accumulate intracellular pools of the α and γ chains, whereas significant intracellular levels of free β chain are not detected. Based on pulse-chase data, the β chain is believed to interact with either the α or γ chain immediately following β chain transport into the ER. When recombinant fibrinogen is expressed at low levels in BHK (baby hamster kidney) cells, intracellular accumulation patterns are similar to those observed with HepG2 cells. However, when the heterologous fibrinogen genes are amplified to high levels in BHK cells, fibrinogen protein assembly and secretion are not significantly increased. Southern analysis confirms the increases in gene copy number. Northern analysis demonstrates an increase in the mRNA levels of all three chains. However, these increases are not reflected in an increase in the amount of correctly assembled multimer present in the secretory pathway or secreted into the medium. The size of the α and γ chain pools increases sigificantly, while β chain continues to be limiting. In addition, surplus α and γ chains appear in a discrete complex which is present intracellularly as well as efficiently secreted. These results suggest the existence of tissue-specific synthesis, transport, or assembly mechanisms in liver-derived HepG2 cells which are not present in BHK cells.

Oral Communication:

XIV Congress of the International Society on Thrombosis and Haemostasis
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